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Inhibition of Subsets of G Protein-coupled Receptors by Empty Mutants of G Protein α Subunits in G_o, G_{11}, and G_{16}*

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We previously reported that the xanthine nucleotide binding $G_o\alpha$ mutant, $G_o\alpha$X, inhibited the activation of G_i-coupled receptors. We constructed similar mutations in $G_{11}\alpha$ and $G_{16}\alpha$ and characterized their nucleotide binding and receptor interaction. First, we found that $G_{11}\alpha$X and $G_{16}\alpha$X expressed in COS-7 cells bound xanthine 5′-*O*-(thiotriphosphate) instead of guanosine 5′-*O*-(thiotriphosphate). Second, we found that $G_{11}\alpha$X and $G_{16}\alpha$X interacted with $\beta\gamma$ subunits in the presence of xanthine diphosphate. These experiments demonstrated that $G_{11}\alpha$X and $G_{16}\alpha$X were xanthine nucleotide-binding proteins, similar to $G_o\alpha$X. Third, in COS-7 cells, both $G_{11}\alpha$X and $G_{16}\alpha$X inhibited the activation of G_q-coupled receptors, whereas only $G_{16}\alpha$X inhibited the activation of G_i-coupled receptors. Therefore, when in the nucleotide-free state, empty $G_{11}\alpha$X and $G_{16}\alpha$X appeared to retain the same receptor binding specificity as their wild-type counterparts. Finally, we found that $G_o\alpha$X, $G_{11}\alpha$X, and $G_{16}\alpha$X all inhibited the endogenous thrombin receptors and lysophosphatidic acid receptors in NIH3T3 cells, whereas $G_{11}\alpha$X and $G_{16}\alpha$X, but not $G_o\alpha$X, inhibited the activation of transfected m1 muscarinic receptor in these cells. We conclude that these empty G protein mutants of $G_o\alpha$, $G_{11}\alpha$, and $G_{16}\alpha$ can act as dominant negative inhibitors against specific subsets of G protein-coupled receptors.

Heterotrimeric G protein signaling pathways are commonly used to transduce signals across cell membranes in eukaryotic cells. G proteins contain three subunits, α, β, and γ, and can be activated by hundreds of seven-transmembrane receptors. Binding of agonist to receptor activates the receptor, which then catalyzes the exchange of GTP for GDP bound to G protein α subunits. Activated GTP-bound α subunits and free $\beta\gamma$ subunits regulate a variety of cellular effectors, including enzymes and ion channels (1–3). G protein α subunits can be divided into four families: G_s, G_i (G_i, G_o, and transducin), G_q (G_q, G_{11}, G_{14}, and G_{16}), and G_{12} (G_{12} and G_{13}). Some G protein-coupled receptors activate only one family of G proteins, whereas other receptors may activate multiple families of G proteins. G_{16} and its mouse homologue G_{15} behave promiscuously; they can be activated by all classes of G protein-coupled receptors (4).

We recently reported that the xanthine nucleotide binding $G_o\alpha$ mutant, $G_o\alpha$X (a double mutant of $G_o\alpha$, D273N/Q205L) can interact with appropriate receptors on the membrane (5, 6). $G_o\alpha$X was regulated by xanthine nucleotides instead of guanine nucleotides. The empty form (nucleotide-free) of $G_o\alpha$X has been shown to form a stable complex with G_o-coupled receptors and to inhibit the cognate receptor by competing with endogenous wild-type G proteins. In the present study, we investigated the functions of similar mutants in another G protein family. We found that both $G_{11}\alpha$X ($G_{11}\alpha$DN/QL) and $G_{16}\alpha$X ($G_{11}\alpha$DN/QL) were xanthine nucleotide-binding proteins. They bound XTPγS, but not GTPγS. These mutant proteins were also able to bind $\beta\gamma$ subunits only in the presence of XDP. In the nucleotide-free state, they interacted with their appropriate receptors and inhibited activation. Furthermore, $G_{11}\alpha$X and $G_{16}\alpha$X retained the same receptor binding specificity of the wild-type proteins. $G_{11}\alpha$X only inhibited G_q-coupled receptors, but not G_i-coupled receptors, whereas $G_{16}\alpha$X was able to inhibit receptors from both families. These results suggest that as with $G_o\alpha$X, $G_{11}\alpha$X, and $G_{16}\alpha$X can be used as dominant inhibitors against a subset of G protein-coupled receptors.

EXPERIMENTAL PROCEDURES

Materials—Purified bovine retinal transducin $\beta\gamma$ were generous gifts from Dr. O. Nakanishi (Division of Biology, Caltech). Xanthine nucleotides and guanine nucleotides were from Sigma. Radioactive [^{35}S]ATPγS,[1] [^{35}S]GTPγS, and [^{3}H]quinuclidinylbenzilate were from NEN Life Science Products.

Mutagenesis of $G_{11}\alpha$ and $G_{16}\alpha$—The D277N mutation was introduced in both wild-type $G_{11}\alpha$ and the activated mutant $G_{11}\alpha$Q209L. The D280N mutation was introduced in both wild-type $G_{16}\alpha$ and the activated $G_{16}\alpha$Q213L. The site-specific mutagenesis was conducted by polymerase chain reaction using oligonucleotide TTCCTCAACAAGAAGGACCTTCTAGAAGAC for $G_{11}\alpha$ and TTTCTCAACAAAACCGACATCCTGGAGGAGAAAATCCC for $G_{16}\alpha$. The cDNAs were subcloned into the pCIS vector under the control of a CMV promoter.

Expression and Purification of His_6-tagged $G_o\alpha$—Both wild-type $G_o\alpha$ and mutant $G_o\alpha$X were subcloned into the *Escherichia coli* expression vector pET-15b (Novagen) with a His_6 tag at the N terminus (5). The recombinant proteins were expressed and purified as described previously. The His_6-tagged proteins were purified over a Ni^{2+}-nitrilotriacetic acid column according to the protocol provided by the manufacturer (Novagen, Inc.). Purified proteins were stored in TED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM dithiothreitol) with 0.1 mM $MgCl_2$.

Membrane Preparation from Baculovirus-infected Sf9 Cells—Sf9 cells were grown and infected with recombinant baculoviruses encoding m2 MAChR (7, 8). Membranes of the infected cells were prepared as described. Infected cells were centrifuged and resuspended at $<10^7$ cells/ml in HME/PI buffer (20 mM NaHepes, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 10 μg/ml leupeptin). The cell suspension was homogenized by 10 strokes in a glass homogenizer followed by passing through a 27-gauge hypodermic needle several times. The homogenate was briefly centrifuged at 3,000 $\times$ *g* for 10 min, and then the supernatant was collected and centrifuged at 30,000 $\times$ *g* for 30 min. The pellet was washed once with HME/PI, and the final pellet was resuspended in HME/PI at a protein concentration of 5 mg/ml.

Synthesis of XTPγS—XTPγS was synthesized from XDP and ATPγS with nucleotide diphosphate kinase as described previously (9). To

[1] The abbreviations used are: ATPγS, adenosine 5′-*O*-(thiotriphosphate); GTPγS, guanosine 5′-*O*-(thiotriphosphate); XTPγS, xanthine 5′-*O*-(thiotriphosphate); MAChR, muscarinic cholinergic receptor; DMEM, Dulbecco's modified Eagle's medium; LPA, lysophosphatidic acid; TRH, thyrotropin-releasing hormone; SRF, serum response factor; SRE, serum response element; PLC, phospholipase.

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produce ^{35}S-labeled XTPγS, the reaction contained 10 μM XDP, 1 μM [^{35}S]ATPγS, and 10 units nucleotide diphosphate kinase (Sigma) in 100 μl of nucleotide diphosphate kinase buffer (1 mM $MgCl_2$, 5 mM dithiothreitol, and 20 mM Tris-HCl, pH 8.0). The mixture was incubated at room temperature for 2 h. The resulting concentration of [^{35}S]XTPγS was about 1 μM (1 μCi/pmol). The radiochemical purity of XTPγS was monitored by TLC on Avicel/DEAE plates (Analtech) in 0.07 N HCl.

Receptor-stimulated GTPγS Binding of Purified $G_o\alpha$—Binding of [^{35}S]GTPγS to recombinant $G_o\alpha$ was performed as described previously (5, 6). 0.5 μg of purified $G_o\alpha$ was first incubated with 1 μg of transducin βγ and 100 μg of m2 MAChR membrane in TED buffer with 10 μM GDP, 0.1 mM $MgCl_2$, and 1 μM ATP for 0.5 h. The reaction was started with the addition of 0.1 μM GTPγS (20,000 cpm/pmol) and 100 μM carbachol. For the time course experiments, 20-μl aliquots were withdrawn from a 200-μl reaction, diluted 10-fold with ice-cold TED buffer containing 0.1 mM $MgCl_2$, filtered through 45-μm nitrocellulose, washed, and dried. The amount of bound radioactivity was determined by scintillation counting.

COS-7 Cell Culture and Transfection—COS-7 cells were cultured in DMEM containing 10% fetal bovine serum. 1×10^5 cells/well were seeded in 12-well plates 1 day before transfection. All transfection assays contained a total amount of 1 μg of DNA, and pCIS encoding β-galactosidase was used to maintain a constant amount of DNA. To each well, 1 μg of DNA was mixed with 5 μl of LipofectAMINE (Life Technologies, Inc.) in 0.5 ml of Opti-MEM (Life Technologies, Inc.), and 5 h later, 0.5 ml of 20% fetal calf serum in DMEM was added to the medium. After 48 h, cells were assayed for inositol phosphate levels as described previously (10, 11).

Immunoprecipitation of XTPγS-bound $G_{11}\alpha X$ and $G_{16}\alpha X$—COS-7 cells were transfected with $G_{11}\alpha$X and $G_{16}\alpha$X 2 days before being lysed in the RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) with 2 mM $MgCl_2$, 1 mM EDTA, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 10 μg/ml leupeptin). The lysate was centrifuged at 12,000 × *g* for 10 min, and the supernatant was incubated with 0.1 μM [^{35}S]XTPγS or [^{35}S]GTPγS (20,000 cpm/ml) for 1 h at room temperature. $G_{11}\alpha$X and $G_{16}\alpha$X proteins were then immunoprecipitated using appropriate antibodies and protein A-Sepharose (Sigma). The amount of radioactive nucleotide in the immunoprecipitates was determined by scintillation counting.

Permeabilization of COS-7 Cell Membranes—Membranes of transfected COS-7 cells were permeabilized as described (5). Cells were washed and incubated in 200 ml of permeabilization solution consisting of 115 mM KCl, 15 mM NaCl, 0.5 mM $MgCl_2$, 20 mM Hepes-NaOH, pH 7, 1 mM EGTA, 100 mM ATP, 0.37 mM $CaCl_2$ (to give a free Ca^{2+} concentration of 100 nM), and 200 units/ml α-toxin with or without 0.1 mM XDP for 10 min at 37 °C. Then 2 μl of 1 M LiCl was added before the inositol phosphate assay.

NIH3T3 Cell Culture and Transfection—NIH3T3 cells were maintained in DMEM containing 10% calf serum. 1×10^5 cells/well were seeded into 24-well plates 1 day before transfection. Total of 1 μg/well of DNA, including 0.2 μg of pSRF-Luc reporter plasmid (Strategene, Inc.), were used to transfect cells with Superfect (Life Technologies, Inc.), following the manufacturer's recommendations. Plasmid of pCIS encoding β-galactosidase was used to maintain a constant amount of DNA for each well.

Luciferase Assay—Transfected NIT3T3 cells were maintained in DMEM containing 0.05% calf serum overnight and then were stimulated with 500 nM of LPA or 1 unit/ml of thrombin in the same medium for 6 h before cell extracts were collected to determine the activity of luciferase. The luciferase assay was performed following the protocol of Luciferase Assay System from Promega. The activity of luciferase was determined by measuring luminescence intensity using a luminometer (Monolight 2010 from Analytical Luminescence Laboratory).

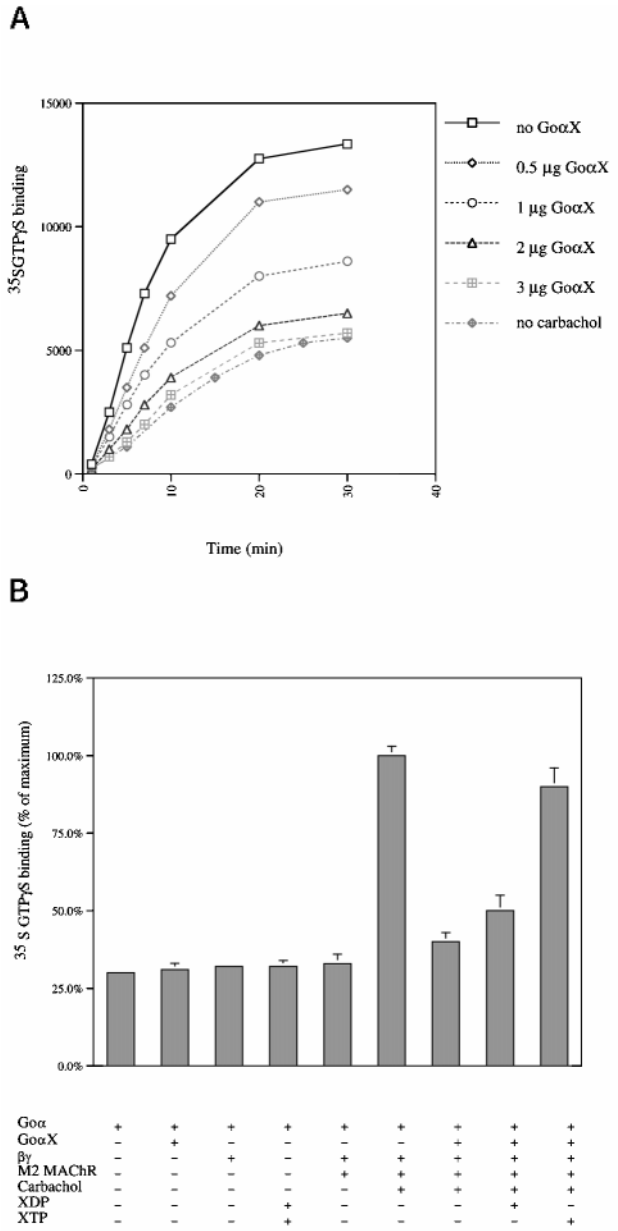

FIG. 1. **$G_o\alpha X$ inhibits the GTPγS binding of wild-type $G_o\alpha$ stimulated by m2 MAChR.** *A*, 100 μg of m2 MAChR membranes was incubated with 0.5 μg of $G_o\alpha$, 1 μg of βγ, 10 μM GDP, and indicated amount of $G_o\alpha$X in TEDM buffer (20 mM Tris-HCl, pH 8.0, 1 mm EDTA, 1 mM dithiothreitol, and 1 mm $MgCl_2$) for 20 min at room temperature and then the mixture was diluted 10-fold with TEDM buffer containing 0.1 μM [^{35}S]GTPγS (20,000 cpm/pmol) and 100 μM carbachol at time 0. 20-μl aliquots were withdrawn and assayed for the bound nucleotides at the indicated times. *B*, 0.5 μg of wild-type $G_o\alpha$ was preincubated with 100 μg of m2 MAChR membranes and 3 μg of $G_o\alpha$X under indicated conditions and then subjected to the similar GTPγS binding assay as in *A*. Only data at 20 min were shown as the percentage of maximum binding.

RESULTS

Inhibition of $G_o\alpha X$ to GTPγS Binding of Wild-type $G_o\alpha$ Stimulated by m2 Muscarinic Receptors—We have previously shown that $G_o\alpha$X inhibited the activation of m2 muscarinic receptors (m2 MAChR) in transfected COS-7 cells (6). To test inhibition of m2 MAChR more directly, we asked whether preincubation of receptor with $G_o\alpha$X inhibited the binding of GTPγS to wild-type $G_o\alpha$ facilitated by activated m2 MAChR *in vitro*. Recombinant m2 MAChR from Sf9 cells has been shown to stimulate the binding of GTPγS to wild-type $G_o\alpha$ 2–3-fold in response to muscarinic agonists (7, 8). We infected Sf9 cells with recombinant baculoviruses encoding m2 MAChR and prepared membranes. The concentration of receptor in isolated membranes was about 20 pmol/mg of membrane protein, determined from [^{3}H]QNB binding. In the control experiments, we reconstituted purified $G_o\alpha$ with the transducin β1γ1 subunits and Sf9 cell membranes containing m2 MAChR and then assayed the binding of GTPγS to $G_o\alpha$ upon activation with the muscarinic agonist carbachol. We found that carbachol accelerated the binding of GTPγS to $G_o\alpha$ (Fig. 1*A*). Then we coincubated different amounts of purified $G_o\alpha$X in similar experi-

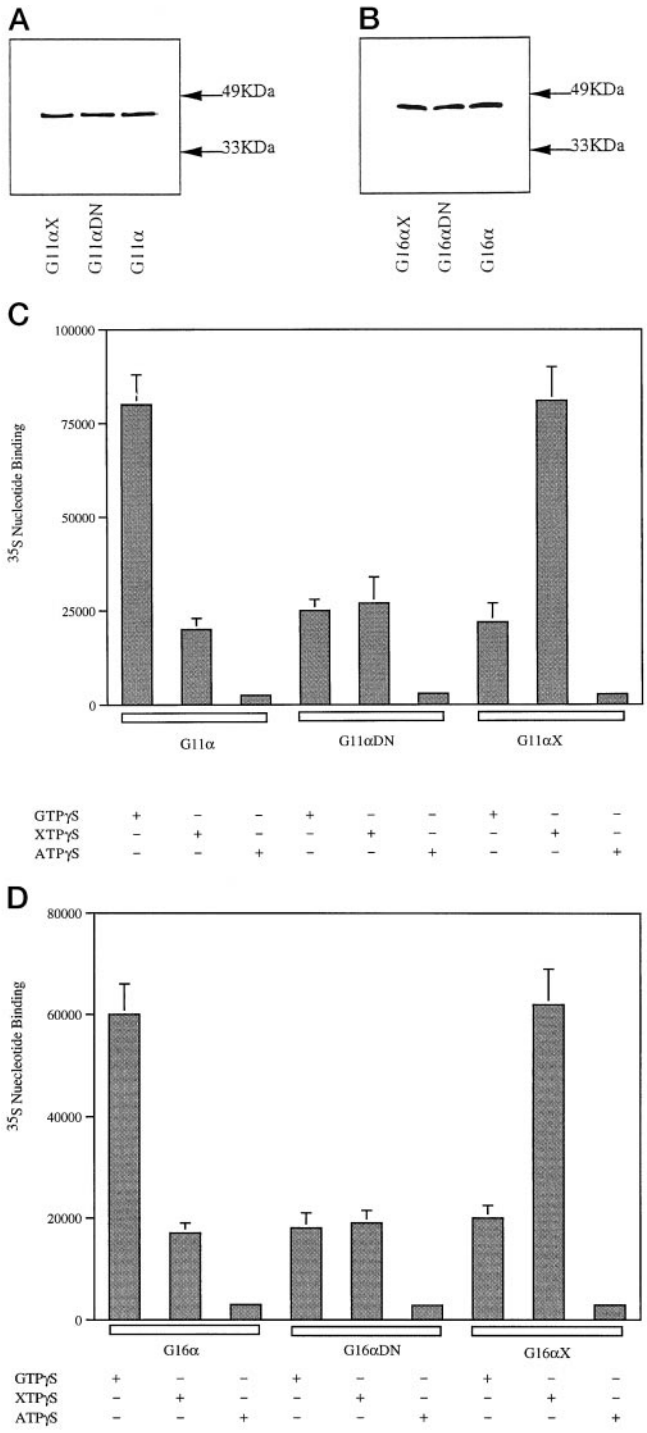

FIG. 2. **XTPγS binding of $G_{11}\alpha X$ and $G_{16}\alpha X$.** In *A* and *B*, mutant proteins of $G_{11}\alpha X$, $G_{11}\alpha DN$, $G_{16}\alpha X$, and $G_{16}\alpha DN$ were expressed in COS-7 cell and subjected to Western blots using antibodies against $G_{11}\alpha$ and $G_{16}\alpha$, respectively. In *C* and *D*, the lysates of transfected COS-7 cells were incubated with indicated radioactive nucleotide for 1 h at room temperature. The mutant protein was then immunoprecipitated using appropriate antibodies and the amount of radioactive nucleotide was determined.

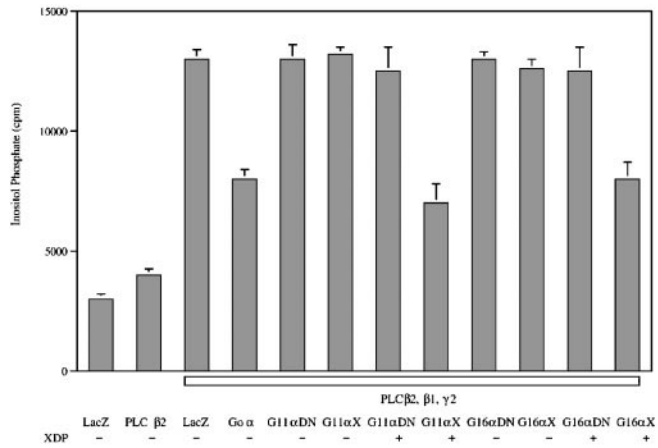

FIG. 3. **The interaction of $G_{11}\alpha X$ and $G_{16}\alpha X$ with $\beta\gamma$ in transfected COS-7 cells is XDP-dependent.** 1×10^5 cells/well were seeded in a 12-well plate and then were transfected with cDNAs encoding the indicated proteins the next day. The total amount of cDNA for each well was adjusted to 1.0 μg by addition of CMV-LacZ cDNA. Cells were labeled with [^{3}H]inositol, and the levels of inositol phosphates were determined after incubating cells with 200 units/ml of α-toxin with or without 10^{-4} M XDP.

ments and found that $G_o\alpha X$ attenuated the m2 MAChR-catalyzed activation of GTPγS binding to $G_o\alpha$. The inhibitory effect of $G_o\alpha X$ was proportional to the amount of $G_o\alpha X$ added (Fig. 1*A*). This result is consistent with our previous finding that $G_o\alpha X$ forms a stable complex with m2 MAChR. Since we have also demonstrated that the interaction between $G_o\alpha X$ and m2 MAChR can be abolished by either XDP or XTP (6), we then tested whether XDP or XTP could relieve the inhibitory effect of $G_o\alpha X$. As expected, $G_o\alpha X$ did not inhibit the activation of m2 MAChR in the presence of XTP. However, $G_o\alpha X$ was able to inhibit the binding of GTPγS to $G_o\alpha$ when XDP was present (Fig. 1*B*). This is not surprising because $G_o\alpha X$ is able to bind $\beta\gamma$ in the presence of XDP. We conclude that $G_o\alpha X$ competes with wild-type $G_o\alpha$ for $\beta\gamma$ subunits and inhibits the activation of $G_o\alpha$ by receptor. This is also consistent with the fact that the activation of $G_o\alpha$ by m2 MAChR requires $\beta\gamma$ (Fig. 1*B*).

Binding of XTPγS to $G_{11}\alpha X$ and $G_{16}\alpha X$—To test whether the DN mutation of the conserved NKXD motif in other G protein α subunits also generates xanthine nucleotide-binding proteins, we introduced the mutation in both wild-type $G_{11}\alpha$ and $G_{16}\alpha$ and into their activated QL mutant cDNA. We then expressed the mutant proteins $G_{11}\alpha DN$ ($G_{11}\alpha D277N$), $G_{11}\alpha X$ ($G_{11}\alpha D277N/Q209L$), $G_{16}\alpha DN$ ($G_{16}\alpha D280N$), and $G_{16}\alpha X$ ($G_{16}\alpha D280N/Q213L$) in COS-7 cells (Fig. 2, *A* and *B*). Unlike $G_o\alpha$, large quantities of active recombinant proteins of $G_{11}\alpha$ and $G_{16}\alpha$ are not easily expressed and purified from *E. coli*. Thus we decided to test the nucleotide binding of the mutant proteins in COS-7 cell lysates. After incubating with the radioactive GTPγS or XTPγS, we immunoprecipitated the mutant proteins and assayed bound radioactive nucleotide. We found that $G_{11}\alpha X$ and $G_{16}\alpha X$ bound XTPγS instead of GTPγS, whereas wild-type $G_{11}\alpha$ and $G_{16}\alpha$ preferred GTPγS (Fig. 2, *C* and *D*). Consistent with our previous finding that $G_o\alpha X$ bound xanthine nucleotides but not guanine nucleotides, whereas $G_o\alpha DN$ did not bind either nucleotides, we found that both $G_{11}\alpha DN$ and $G_{16}\alpha DN$ did not show strong binding of either [^{35}S]GTPγS or [^{35}S]XTPγS.

XDP-dependent $\beta\gamma$ Interaction of $G_{11}\alpha X$ and $G_{16}\alpha X$—We previously showed that $G_o\alpha X$ was able to bind $\beta\gamma$ subunits in the presence of XDP (5). To test whether $G_{11}\alpha X$ and $G_{16}\alpha X$ also shows XDP-dependent $\beta\gamma$ interaction, we assayed their binding with $\beta\gamma$ in transfected COS-7 cells. In COS-7 cells, $\beta1\gamma2$ is able to activate PLCβ2, and the activation of PLCβ2 can be inhibited by cotransfection with wild-type $G_o\alpha$ because of competition for $\beta\gamma$ (5, 12). We cotransfected COS-7 cells with PLCβ2, $\beta1$, $\gamma2$, and $G_{11}\alpha X$ or $G_{16}\alpha X$ and found that neither mutant inhibited PLCβ2 activity, whereas wild-type $G_o\alpha$ did inhibit. This experiment suggests that $G_{11}\alpha X$ and $G_{16}\alpha X$ do not bind $\beta\gamma$

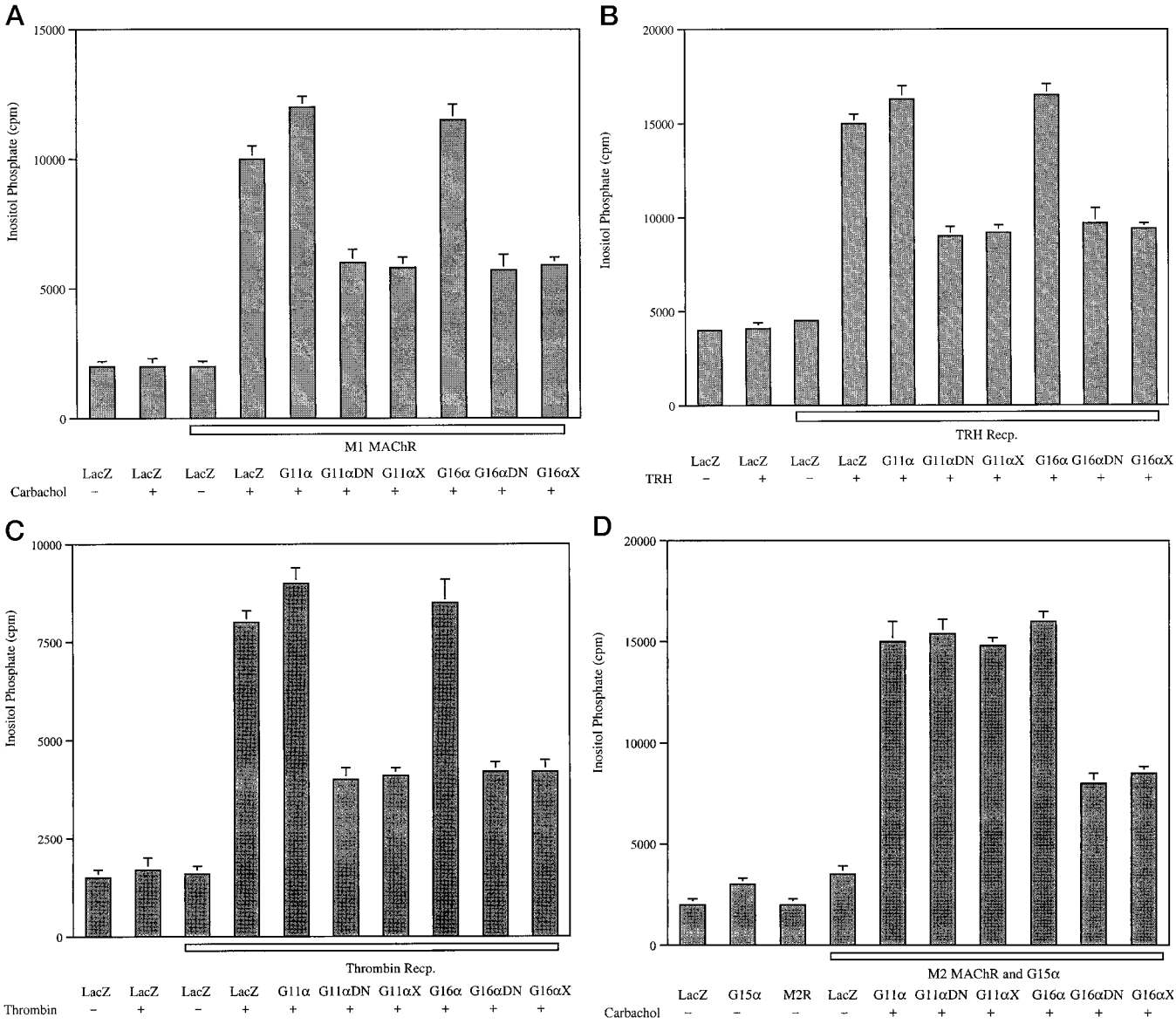

FIG. 4. **Empty mutants of $G_{11}\alpha$ and $G_{16}\alpha$ inhibited appropriate receptors in COS-7 cells.** 1×10^5 cells/well were seeded in a 12-well plate and then transfected with m1 MAChR (*A*), TRH receptor (*B*), thrombin receptor (*C*), or m2 MAChR (*D*) and indicated Gα. In *A–C*, the amount of receptor cDNA for each well was 0.25 μg, and the amount of Gα was 0.75 μg. In *D*, the amount of both m2 MAChR and $G_{15}\alpha$ cDNA was 0.2 μg/well and that of mutant Gα was 0.6 μg/well. After cells were labeled with [^{3}H]inositol overnight, they were incubated for 30 min in the medium containing 1 μM carbachol (*A* and *D*), 1 μM TRH (*B*), or 0.1 unit/ml thrombin (*C*) before levels of inositol phosphates were determined.

presumably because the intracellular concentration of XDP is negligible (13) (Fig. 3). To deliver XDP into cells, we permeabilized the cell membrane with α-toxin. After incubating transfected COS-7 cells with α-toxin in the presence of XDP, we found that both $G_{11}\alpha$X and $G_{16}\alpha$X inhibited PLCβ2 activity stimulated by $\beta\gamma$ (Fig. 3). In the similar experiments with $G_{11}\alpha$DN and $G_{16}\alpha$DN, we did not see inhibition of the activation of PLCβ2, even when XDP was present (Fig. 3). In the control experiments, we found that α-toxin alone or α-toxin followed by GDP or GTP addition did not affect the activity of PLCβ2 (data not shown). These experiments show that $G_{11}\alpha$X and $G_{16}\alpha$X do not bind $\beta\gamma$ and interfere with its activation of PLCβ2 in the nucleotide-free state. However, when in the XDP-bound form, $G_{11}\alpha$X and $G_{16}\alpha$X are able to sequester cellular $\beta\gamma$ and inhibit its ability to activate PLCβ2.

Dominant Negative Inhibition of G Protein-coupled Receptor by Empty $G_{11}\alpha$X and $G_{16}\alpha$X—$G_o\alpha$X binds to members of the G_i-coupled receptor family and has been shown to act as a dominant inhibitor of G_i-coupled receptors (6). To test receptor interaction of $G_{11}\alpha$X and $G_{16}\alpha$X, we assayed their ability to inhibit the activation of G protein-coupled receptors in COS-7 cells. m1 muscarinic receptors (m1 MAChR) have been shown to primarily activate the Gq family of G proteins (10). Activated $G_q\alpha$ then stimulates PLCβ isoforms to elevate cellular inositol 1,4,5-trisphosphate concentration. We cotransfected COS-7 cells with m1 MAChR and $G_{11}\alpha$X or $G_{16}\alpha$X and tested whether the mutant proteins inhibited the activation of m1 MAChR by competing with endogenous $G_q\alpha$. We found that both $G_{11}\alpha$X and $G_{16}\alpha$X were able to inhibit the activity of endogenous PLCβ isoforms stimulated by activated m1 MAChR (Fig. 4*A*). Since $G_{11}\alpha$X and $G_{16}\alpha$X did not affect the activation of PLCβ2 by $\beta\gamma$ in COS-7 cells, the inhibition of m1 MAChR-stimulation PLCβ activation by $G_{11}\alpha$X and $G_{16}\alpha$X most probably results from the competitive binding of the mutant proteins to the receptor. In similar experiments using $G_{11}\alpha$DN and $G_{16}\alpha$DN, we found that they inhibited the activation of m1 MAChR as well as $G_{11}\alpha$X and $G_{16}\alpha$X (Fig. 4*A*), suggesting that $G_{11}\alpha$DN and $G_{16}\alpha$DN could also bind to m1 MAChR, although they do not bind either

guanine nucleotides or xanthine nucleotides. In the experiments with two other G_q-coupled receptors, TRH receptor and thrombin receptor (14–16), we found that all four mutant proteins were able to inhibit activation by receptors (Fig. 4, *B* and *C*). These results agree with our previous observation that the empty $G_o\alpha$ mutant protein forms a stable complex with appropriate receptor. Thus our working hypothesis is that in the absence of xanthine nucleotides sufficient levels of mutant proteins are made to interact with the appropriate receptors.

Empty $G_o\alpha$X mutants appear to retain the receptor binding specificity of wild-type $G_o\alpha$ (6). To test whether empty $G_{11}\alpha$ and $G_{16}\alpha$ mutants also behave similarly, we assayed for their inhibition of activation by the m2 MAChR, a member of G_i-coupled receptor family. Since m2 MAChR couples only to the G_i family of Gα proteins, but not to the G_q family (4, 9), we could not assay the activity of m1 MAChR by monitoring $G_q\alpha$-stimulated PLCβ activities in COS-7 cells. Therefore we constructed an artificial pathway by cotransfecting both m2 MAChR and $G_{15}\alpha$ into COS-7 cells. $G_{15}\alpha$ and $G_{16}\alpha$ are homologous proteins (G_{15} mouse and G_{16} human) that behave as a promiscuous G protein, which can be activated by all kinds of G protein-coupled receptors, and activated G_{15} stimulates the activity of PLCβ (4). In cells cotransfected with both m2 MAChR and $G_{15}\alpha$, we were able to activate endogenous PLCβ isoforms by the addition of the muscarinic agonist carbachol. In the cotransfection experiments with m2 MAChR, $G_{15}\alpha$, and the empty mutants of $G_{11}\alpha$ and $G_{16}\alpha$, we found that only $G_{16}\alpha$X and $G_{16}\alpha$DN inhibited activation by m2 MAChR, whereas $G_{11}\alpha$X and $G_{11}\alpha$DN had no effect (Fig. 4*D*). This is consistent with the fact that $G_{11}\alpha$ does not couple to the m2 MAChR and $G_{16}\alpha$ does. These experiments suggest that both empty mutants of $G_{11}\alpha$ and $G_{16}\alpha$ retain the binding specificity of their wild-type counterparts; $G_{11}\alpha$X and $G_{11}\alpha$DN only interact with G_q-coupled receptors, but not with G_i-coupled receptor, whereas $G_{16}\alpha$X and $G_{16}\alpha$DN can interact with both families of receptors.

Inhibition of Endogenous Thrombin Receptor and LPA Receptor in NIH3T3 Cells—NIH3T3 cells express endogenous thrombin receptors and LPA receptors (17). These two types of receptors couple to a variety of G proteins, including G_i, G_q, and $G_{12/13}$ (16, 18). Activation of these receptors leads to the activation of serum response factor (SRF) and SRF-mediated gene transcription through RhoA, via both the G_q and $G_{12/13}$ pathways presumably. To investigate whether empty Gα mutants inhibit thrombin receptor and LPA receptor in NIH3T3 cells, we determined the activity of luciferase, whose expression was under the regulation of SRE.L, when the cells were cotransfected with the mutant G proteins and the reporter gene plasmid. SRE.L is a derivative of the c-Fos serum response element (SRE) to which SRF binds and activates luciferase transcription (19). We found that the empty mutants of all three types of a subunits, $G_o\alpha$, $G_{11}\alpha$, and $G_{16}\alpha$, were able to inhibit the activation of both thrombin receptors (Fig. 5*A*) and LPA receptors (Fig. 5*B*). These results are consistent with the experiments in COS-7 cells showing that the empty G proteins bind tightly to their cognate receptors. To exclude the possibility that empty Gα mutants interfere with the downstream components of the signaling pathway, we cotransfected the cells with the constitutively activate G_q mutant, $G_q\alpha$QL, and the empty Gα mutants. We found that the empty Gα mutants did not affect $G_q\alpha$QL-stimulated SRF activation (data not shown), indicating that the inhibition of receptor-stimulated SRF activation by empty Gα mutants must come from their competitive binding to the receptor, not from direct Gα activation of downstream effectors.

NIH3T3 cells apparently do not have endogenous muscarinic receptors, since addition of carbachol did not lead to the activation of SRF (17). We transfected the cells with m1 MAChR and found that its activation resulted in stimulated luciferase activity, presumably through the endogenous G_q pathway. Co-

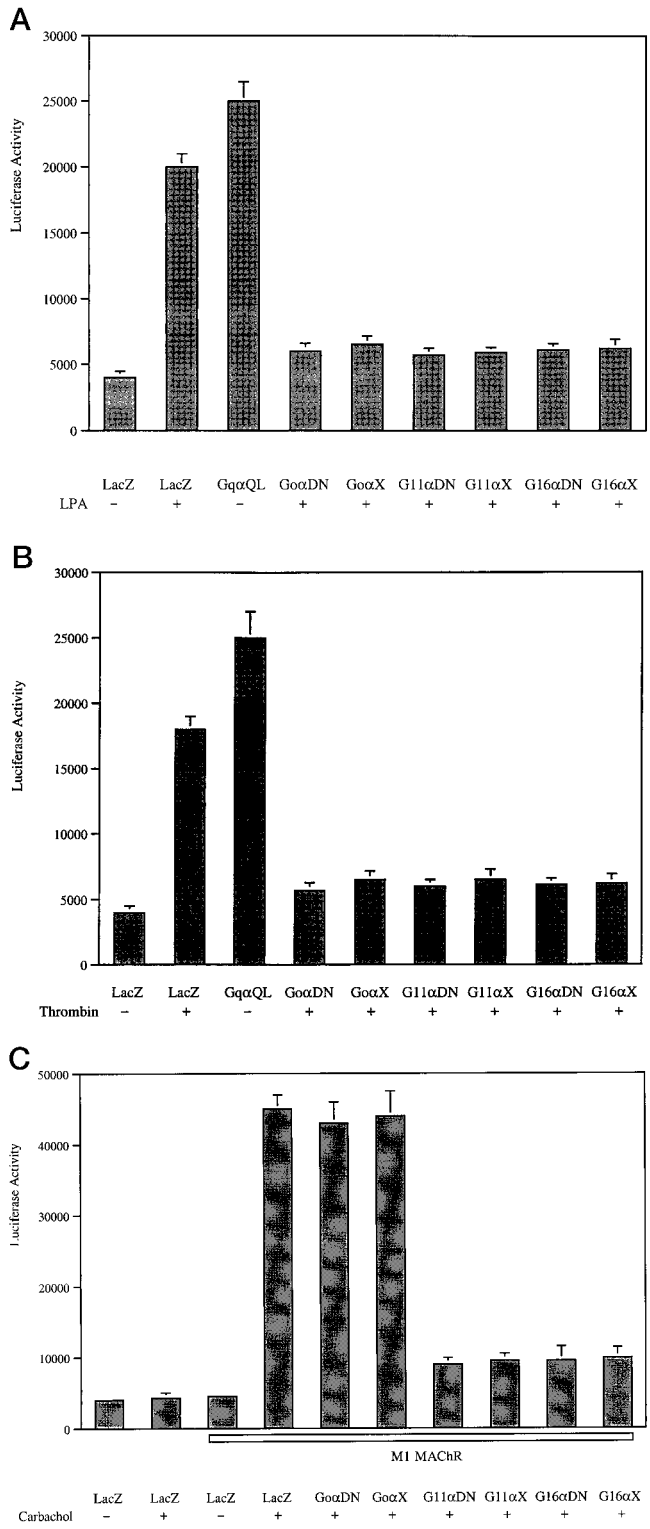

FIG. 5. **Empty mutants of $G_o\alpha$, $G_{11}\alpha$, and $G_{16}\alpha$ inhibited endogenous thrombin and LPA receptors in NIH3T3 cells.** 1×10^5 cells/well were seeded in a 24-well plate and then transfected with 0.2 μg of SRF-luciferase reporter plasmid cDNA and 0.75 μg of indicated Gα mutant cDNA (*A* and *B*) or 0.2 μg of SRF-luciferase reporter plasmid cDNA, 0.2 μg of m1 MAChR cDNA, and 0.5 μg of indicated Gα mutant cDNA (*C*). After 24-h starvation, cells were stimulated with 0.5 μM LPA (*A*), 1 unit/ml thrombin (*B*), or 1 μM carbachol (*C*) for 6 h before the activity of luciferase was determined.

expression of the Gα mutants showed that $G_{11}\alpha$X, $G_{11}\alpha$DN, $G_{16}\alpha$X, and $G_{16}\alpha$DN inhibited the activation of m1 MAChR, but $G_o\alpha$X and $G_o\alpha$DN did not. These experiments indicate that the presumptive empty form of $G_{11}\alpha$ and $G_{16}\alpha$ bound m1 MAChR whereas the empty form of $G_o\alpha$ did not, consistent with the results from COS-7 cell experiments.

DISCUSSION

We previously reported that $G_o\alpha$X, the xanthine nucleotide-binding mutant protein of $G_o\alpha$, formed stable complexes with their appropriate receptors and inhibited the activation of cognate receptors because of competitive binding (6). In this study, we reconstituted $G_o\alpha$X, $G_o\alpha$, $\beta\gamma$, and m2 MAChR and Sf9 cell membranes. We monitored the GTPγS binding of $G_o\alpha$ facilitated by m2 MAChR upon the activation of its agonist carbachol. Not surprisingly, we found that $G_o\alpha$X inhibited the nucleotide exchange of wild-type $G_o\alpha$ catalyzed by the activated m2 MAChR. Therefore, we demonstrated that $G_o\alpha$X was able to inhibit the activation of m2 MAChR *in vitro*.

To extend this work to other families of G proteins, we introduced the similar DN mutation in wild-type $G_{11}\alpha$ and $G_{16}\alpha$, as well as activated $G_{11}\alpha$QL and $G_{16}\alpha$QL, and expressed the mutant proteins in COS-7 cells. After immunoprecipitating the mutant proteins incubated with radioactive nucleotides in COS-7 cell lysates, we found that $G_{11}\alpha$X and $G_{16}\alpha$X bound XTPγS instead of GTPγS, whereas wild-type $G_{11}\alpha$ and $G_{16}\alpha$ preferred GTPγS. However, $G_{11}\alpha$DN and $G_{16}\alpha$DN did not appear to bind either nucleotides. We also showed that the mutant proteins of $G_{11}\alpha$X and $G_{16}\alpha$X expressed in COS-7 cells interacted with $\beta\gamma$ subunits in a XDP-dependent fashion; they only bound $\beta\gamma$ when XDP was available, whereas $G_{11}\alpha$DN and $G_{16}\alpha$DN did not. These results are consistent with previous findings using $G_o\alpha$X and $G_o\alpha$DN; the single DN mutation resulted in a loss of ability to bind nucleotides, whereas the double DN/QL mutation lead to xanthine nucleotide binding (5). Although the mutation of Asp $\rightarrow$ Asn in the conserved NKXD motif of G protein α subunits was expected to switch the nucleotide specificity of the mutated protein from guanine nucleotide to xanthine nucleotide, according to the available crystal structures of G protein α subunits and other GTP-binding proteins, we observed that the single DN mutation resulted in a protein not able to bind either nucleotides in three G protein α subunits: $G_o\alpha$, $G_{11}\alpha$, and $G_{16}\alpha$. It is not apparent from the crystal structures why the second QL mutation, a well characterized GTPase-deficient mutation, restored the xanthine nucleotide binding of the mutant proteins; the conserved Gln (position 200 in transducin α) resides at the opposite side of the nucleotide binding pocket from the DN mutation (position 268 in transducin α).

To test whether empty mutants of $G_{11}\alpha$ and $G_{16}\alpha$ interacted with G protein-coupled receptors and inhibited the activation of appropriate receptors, we assayed the stimulated PLCβ activity by transfected receptors in COS-7 cells and the activation of SRF by endogenous thrombin receptors and LPA receptors in NIH3T3 cells. We found that $G_{11}\alpha$X and $G_{16}\alpha$X inhibited the activation of m1 of MAChR and TRH receptor, but not m2 MAChR, whereas $G_o\alpha$X and $G_o\alpha$DN inhibited the activation of m2 MAChR, but not m1 MAChR or TRH receptor. Furthermore, $G_{16}\alpha$X and $G_{16}\alpha$DN were found to inhibit the activation of m1 MAChR, TRH receptor, and m2 MAChR, in addition to that $G_o\alpha$X, $G_o\alpha$DN, $G_{11}\alpha$X, $G_{11}\alpha$DN, $G_{16}\alpha$X, and $G_{16}\alpha$DN were all able to inhibit the activation of thrombin receptors and LPA receptors. Therefore, we conclude that these empty mutants of G protein α subunits retain the same receptor binding specificity of their wild-type counterparts. Empty $G_o\alpha$ interacts with only G_i-coupled receptors, and empty $G_{11}\alpha$ interacts with only G_q-coupled receptors, while $G_{16}\alpha$ can interact with both families of G protein-coupled receptors. It is interesting to note that $G_{11}\alpha$DN and $G_{16}\alpha$DN were able to inhibit the activation of their appropriate receptors as effectively as $G_{11}\alpha$X and $G_{16}\alpha$X, although $G_{11}\alpha$DN and $G_{16}\alpha$DN did not bind xanthine nucleotides. Similarly, $G_o\alpha$DN was shown to interact with receptors but not able to bind nucleotides (5, 6). These experiments proved that the empty mutant forms of these G protein α subunits can act as effective dominant negative inhibitors against a subset of G protein-coupled receptors. They can be very useful tools to dissect signaling pathways of different G protein-coupled receptors by specifically blocking one family of receptors.

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